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$CD8^+TCR^+$ and $CD8^+TCR^-$ Cells in Whole Bone Marrow Facilitate the Engraftment of Hematopoietic Stem Cells across Allogeneic Barriers

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Summary

Although purified hematopoietic stem cells (HSC) are sufficient to engraft irradiated allogeneic recipients, bone marrow (BM) contains other cells that facilitate engraftment. Here, several candidate facilitators were tested by cotransplantation with HSC. Both TCR^+ and $TCR^-CD8\alpha^+$ BM subpopulations have facilitative potential. $CD8^+TCR^+$ cells are typical T lymphocytes. $CD8^+TCR^-$ facilitators are $CD3^-$, not $CD3^+$, have a granular morphology, and are $CD8\beta^-$ and $CD11c^+$; they share phenotypic characteristics with $CD8\alpha\alpha$ lymphoid dendritic cells and veto cells. We also demonstrate that lytic function is not necessary for facilitation and that the $CD8\alpha$ molecule is either important for facilitation or in the development of facilitators.

Introduction

The search for regimens that would allow long-term multilineage engraftment of hematopoietic cells between allogeneic individuals without severe complications has been a focus of hematological and immunological research for decades. Allogeneic bone marrow engraftment has, unfortunately, been accompanied by graft-versus-host disease (GVHD), wherein donor T cells are involved in an often lethal multisystem immune response against host tissues. Preclinical (Rodt et al., 1974; Korngold and Sprent, 1978) and clinical (Filipovich et al., 1982; Prentice et al., 1982) studies suggested that GVHD could be averted by depletion of T cells in the hematopoietic graft. It soon became evident, however, that this had adverse consequences; namely, grafts depleted of T cells had a decreased engraftment capacity (reviewed in Martin and Kernan, 1990). With the purification of HSC in mice (Spangrude et al., 1988), the quantitative aspects of the engraftment barrier in lethally irradiated allogeneic recipients could be studied (Shizuru et al., 1996; Wang et al., 1997; Gandy and Weissman, 1998; Uchida et al., 1998). Whereas 100 $c\text{-}Kit^+Thy1.1^{lo}Lin^{-/lo}Sca\text{-}1^+$ (KTLS) HSC provide radioprotection for 95%–100% (PD95) of lethally irradiated Ly5-congenic histocompatible hosts, 1,000–2,000 KTLS are required for radioprotection of H-2 mismatched allogeneic recipients (Gandy and Weissman, 1998; Uchida et al., 1998). Although it requires 10- to 20-fold more allogeneic than congenic cells to achieve the PD95 in a given setting, only 2-fold more cells are required to reach given engraftment parameters at high doses: 5,000 congenic or 10,000 allogeneic HSC are required to obtain WBC and platelet engraftment by 10–11 days (Uchida et al., 1998). Even at high doses of HSC, no mice develop GVHD. In contrast to the congenic setting, non-HSC elements in the BM graft appear to have a role in allogeneic radioprotection and donor-derived engraftment. Whereas 100 HSC and 2×10^5 BM (the amount of BM containing 100 HSC) can fully radioprotect and engraft congenic recipients, 500 HSC do not radioprotect to the degree of 1×10^6 BM in allogeneic recipients (10%–40% survival versus 100%). The cells responsible for the differential engraftment between the HSC and BM populations in the allogeneic setting are the focus of this study.

Over the last decade, much effort has focused on characterization of cells expressing T cell markers with the hope of identifying a population that facilitates the engraftment of hematopoietic cells across allogeneic barriers without causing GVHD. There is a long and well-established precedence for the existence of facilitative populations (Storb et al., 1968; Deeg et al., 1979; Ildstad et al., 1986; Sykes et al., 1988). Similarly, there is a long and well-established precedence for different hematopoietic populations having facilitative potential (Storb et al., 1968; Deeg et al., 1979; Lapidot et al., 1992; Murphy et al., 1992; Martin, 1993; Kaufman et al., 1994; Palathumpat et al., 1995; Drobyski and Majewski, 1997). Lapidot et al. demonstrated that $CD8^+CD4^-$ thymocytes could enhance the engraftment of T cell–depleted marrow in the apparent absence of graft-versus-host disease (Lapidot et al., 1992). Martin et al. subsequently demonstrated that $CD8^+$ cells from peripheral lymph node (PLN) could facilitate the engraftment of T cell–depleted marrow across both major and minor histocompatibility barriers in irradiated recipients (Martin, 1993). They were able to identify a dose of $CD8^+$ cells at which facilitation was observed but at which only mild and transient GVHD was detected. Clinical translation of this principle, however, has been limited; in a recent trial in which $CD8^+$ cells were purified by negative depletion of $CD4^+$ cells, a dose of $CD8^+$ cells that would allow facilitation without causing GVHD was not identified (Martin et al., 1999). The reason for this is not yet clear.

Therefore, a search for a population of cells that facilitates without causing GVHD continues. Much effort has been directed at BM $CD8^+$ subpopulations. Kauffman et al. studied subpopulations of BM for their ability to facilitate the engraftment of hematopoietic progenitors across allogeneic barriers (Kaufman et al., 1994). These studies found facilitator potential in $CD8\alpha^+CD8\beta^+TCR\beta^-TCR\gamma\delta^-CD3^+$ cells but not in $CD8^+TCR^+CD3^+$ cells. This contrasted with previous results in which infusion of $CD8^+$ cells from various sources (Lapidot et al., 1992; Martin, 1993) (almost all of which are TCR^+) had facilitative potential. The studies described here were designed to use purified HSC and facilitators in physiological doses. The results confirm that $CD8^+$ cells

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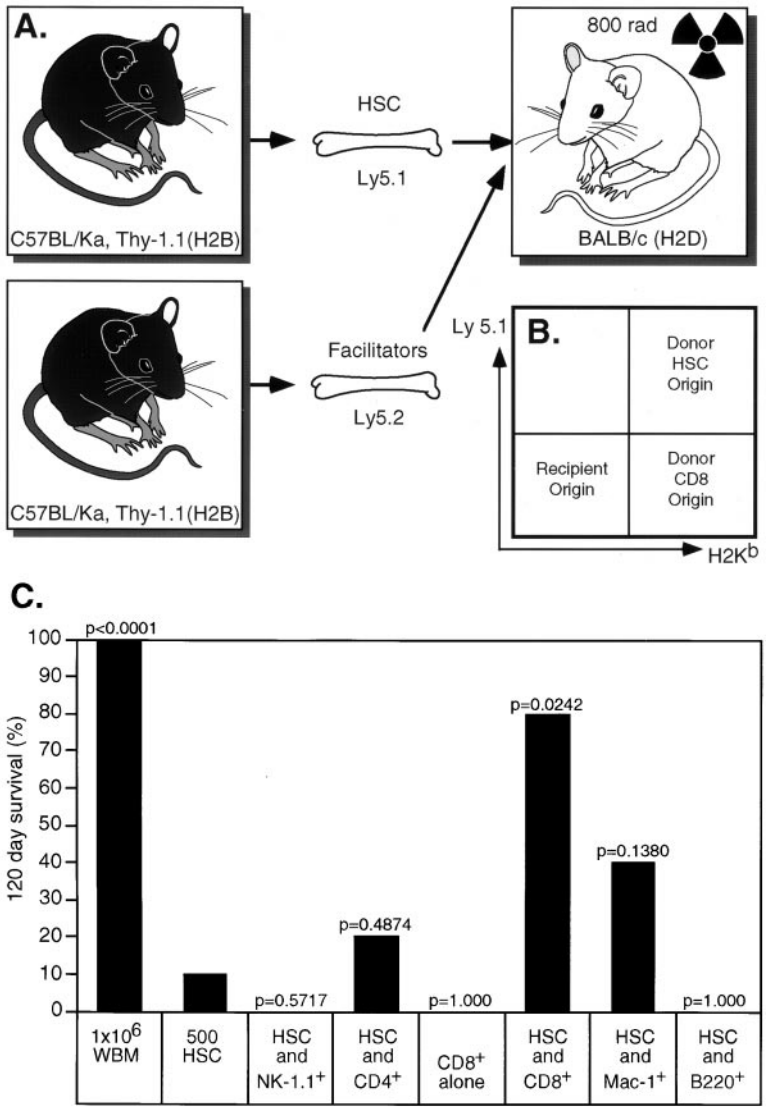

Figure 1. Model and Evaluation of Populations of BM for Facilitator Potential

(A) Description of the model. HSC are purified from C57BL/6, Thy-1.1 (H-2^b) mice. Subpopulations of BM are sorted from mice of the same haplotype, which differ at the allelic Ly5 (CD45) marker. Mice are coinjected with 500 HSC and the number of cells in the specific subpopulation that are found in 1×10^6 BM. Recipient mice are lethally irradiated.

(B) Schemata for analysis of reconstitution. As evident in (A) and as previously described, this system allows cells of HSC, facilitator, and host origin to be differentiated from one another as demonstrated on the two dimensional plot. All analysis is done with PI staining.

(C) Survival at 120 days in lethally irradiated reconstituted mice. Mice received BM, HSC, or HSC and a subpopulation of BM as indicated. One group of mice received $CD8^+$ cells alone as controls. The p values compare experimental groups with mice receiving HSC alone.

facilitate when used in the amounts contained in 1×10^6 BM and that facilitation can be detected with dosages at which GVHD is not found. These studies also demonstrate that facilitative potential is found in both $CD8^+$ TCR^-CD3^- and $CD8^+TCR^+CD3^+$ populations. However, only rare $CD8^+TCR^-CD3^+$ cells could be found in 1×10^6 C57BL/Ka BM, and facilitation could not be detected using the numbers present in 1×10^6 BM. Finally, facilitator cells were depleted in BM of $CD8\alpha^{-/-}$ mice but were replete in mice whose $CD8^+$ and NK killer function was depleted. The latter findings suggest that the $CD8\alpha$ molecule but not lytic function is important in facilitation and/or important in the development of the potential to facilitate.

Results

$CD8^+$ Cells Enhance Survival of Mice Reconstituted with Limiting Numbers of Allogeneic HSC

The experimental model is described in detail in Experimental Procedures. It is based on the hypothesis that non-HSC populations within BM facilitate the engraftment of purified HSC across allogeneic barriers. Early studies have shown that mice reconstituted with 500 allogeneic Thy-$1.1^{lo}Lin^{-/lo}Sca\text{-}1^+$ (TLS) HSC had lower survival rates than mice reconstituted with an amount of BM known to contain this number of TLS cells (Shizuru et al., 1996), implying that this model could serve as the basis for identifying the facilitative potential described in other model systems. In this study, to identify the populations within BM that had the ability to enhance the engraftment of purified HSC across allogeneic barriers, $CD4^+$, $CD8^+$, $B220^+$, NK-1.1^+, and Mac-1^+ populations were isolated by FACS from Ly 5.2, C57BL/Ka donors and coinjected with HSC from Ly 5.1, C57BL/Ka donors (Figure 1A). As shown in Figure 1B, three populations (HSC, putative facilitator, and host) and their progeny can be differentiated in reconstituted mice by cell surface marker staining. Figure 1C shows the survival data from these experiments. Ten percent of mice given 500 TLS alone survived; 100% of mice given 1×10^6 BM (which contains approximately this number of HSC) survived. The only subpopulation of BM

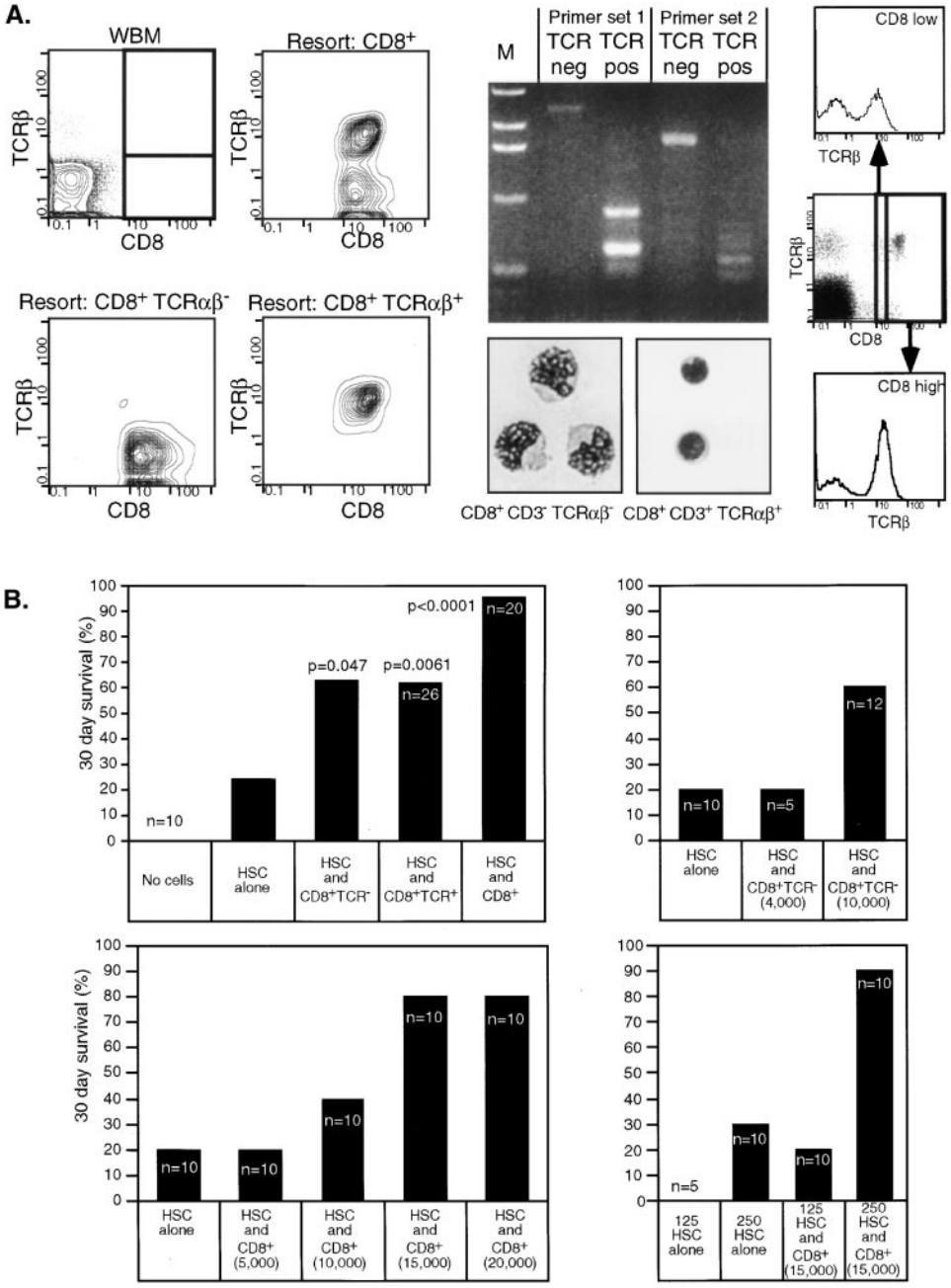

Figure 2. The CD8α$^+$ Population Can Be Divided into a TCRβ$^-$ and a TCRβ$^+$ Population with Facilitator Potential

(A) Top left panel shows BM stained for CD8α and TCRβ. CD8α$^+$, CD8$^+$TCR$^+$, and CD8$^+$TCR$^-$ cells were double sorted, with some of each sample being collected for reanalysis (top left panels), some being sorted directly into PCR tubes for the PCR analysis (top middle panel), and some being collected for May-Grünwald-Giemsa staining (lower middle panel). Right panels show the TCRβ staining of CD8^{lo} versus CD8^{hi} populations of BM.

(B) Top left, host mice were reconstituted with 250–500 KTLS HSC alone, 250–500 KTLS HSC and CD8$^+$TCR$^-$ cells, 250–500 KTLS HSC and CD8$^+$TCR$^-$ cells, and 250–500 KTLS HSC and CD8^{tot} cells. Thirty day survival is shown. P values compare with mice receiving HSC alone. Top right: host mice were reconstituted with 250 KTLS HSC alone and 250 KTLS HSC plus two different dosages of CD8$^+$TCR$^-$ cells. Bottom left: host mice were reconstituted with 250 KTLS HSC alone and 250 KTLS HSC plus four different dosages of CD8^{tot} cells. Bottom right: host mice were reconstituted with 125 or 250 KTLS HSC with or without 15,000 CD8^{tot} cells. All hosts: lethally irradiated BALB/c.

that enhanced survival as determined by Fisher's exact analysis compared with TLS alone was the CD8$^+$ subfraction (1.6×10^4 cells/injection). No mice given CD8$^+$ (1.6×10^4 cells/injection) cells alone survived, consistent with previous studies showing no HSC activity in the CD8$^+$ compartment (Uchida and Weissman, 1992).

The CD8$^+$ Populations Can Be Divided into TCR$^+$ and TCR$^-$ Populations

The CD8$^+$ population comprised 0.8%–1.8% of BM from 4- to 8-week-old mice and could be divided into CD8$^+$TCR$^-$ and CD8$^+$TCR$^+$ populations by FACS (Figure 2A). The TCR$^+$:TCR$^-$ ratio ranged from 3:1 to 6:1.

The TCRβ status of these populations was confirmed by PCR. As expected, the TCRβ genes were in the germline configuration in the cells sorted as TCR^-, whereas the TCR^+ cells yielded characteristic TCRβ rearrangement products. Morphology of the cells was evaluated by May-Grünwald-Giemsa stain (Figure 2A). The TCR^+ population resembles typical T lymphocytes. The TCR^- population is heterogeneous, but most cells were characterized by a granular cytoplasm and a low nuclear/cytoplasm ratio. Despite the fact that these cells appeared to be larger than typical lymphocytes, these populations were not readily separated by forward or side scatter parameters. Both were contained within standard "lymphoid gates." It is of note that the modal CD8α fluorescence of the TCR^- compartment was lower than that of the TCR^+ population (Figure 2A).

The $CD8^+TCR^+$ and $CD8^+TCR^-$ Subpopulations of BM Enhance the Survival of Mice Reconstituted with Limiting Numbers of KTLS

$CD8^+TCR^-$ and $CD8^+TCR^+$ cells were evaluated for their ability to facilitate HSC engraftment across allogeneic barriers in a survival assay. In subsequent experiments KTLS HSC (Ikuta and Weissman, 1992; Uchida et al., 1998) were used. As previously described, the survival of mice allogeneically reconstituted with double-sorted KTLS cells was improved over that found with single-sorted TLS cells (Gandy and Weissman, 1998); survival with KTLS ranges from 10%–40% after reconstitution with 250 HSC. As shown in Figure 2B, both the $CD8^+TCR^-$ and the $CD8^+TCR^+$ subpopulations enhanced survival. Mice were given numbers of KTLS and $CD8^+$ cells that approximated the numbers in 1×10^6 BM. However, to achieve statistically significant facilitation with the $CD8^+TCR^-$ cells, it was necessary to increase the cell dose. When either the $TCR\alpha\beta^+$ or $TCR\alpha\beta^-$ subpopulation of $CD8^+$ cells was administered with the KTLS population in a given experiment, the survival was less than that obtained when the total $CD8^+$ population was given with KTLS.

There is a threshold number of $CD8^+$ cells and a threshold number of HSC below which facilitation is not observed (Figure 2B). In the colony of mice evaluated, the numbers were 10,000 $CD8^+TCR^-$ cells or 15,000 $CD8^{tot}$ ($CD8^+TCR^+$ and $CD8^+TCR^-$). Similarly, enough facilitation to enhance survival was not detected with less than 250 HSC.

The $CD8^{tot}$, $CD8^+TCR^+$, and $CD8^+TCR^-$ Populations of BM Enhance the Reconstitution by KTLS-Derived Populations in Allogeneic Reconstitution

Reconstitution data from PBMC analysis of the surviving mice in one experiment are presented in Table 1. Survivors are all reconstituted with predominantly HSC-derived cells, although the levels of donor-derived cells are lower in the populations reconstituted with KTLS alone and KTLS + $CD8^+TCR^-$ cells (88.5% and 82% ± 19.5% when compared with mice reconstituted with KTLS + $CD8\alpha^+TCR^+$ and KTLS + $CD8\alpha^{tot}$ (98% ± 2.4% and 99% ± 0.4%). Over 99% of Mac-1^+ cells in all recipient groups are of donor origin. Reconstitution differences are most notable in analysis of the $B220^+$ and $CD3^+$ populations. Whereas 78.5% of $B220^+$ cells are of donor

Table 1. Successful Facilitation of Allogeneic HSC Transplants Is Associated with Elimination of Host $CD3^+$ Cells in the Blood

Reconstitution at 8 Weeks	H-$2K^b$ Percent of Total	Mac-1 Percent of Total	Percent Mac-1 HSC-Derived	B220 Percent of Total	Percent B220 HSC-Derived	CD3 Percent of Total	Percent CD3 HSC-Derived	Percent CD3 Host-Derived	Different from HSC Alone
C57Bl/Ka Control	99.7	49.0	98.2	36.0	99.8	29.5	**99.6**	NA	NA
BALB/c Control	0.1	33.1	1.0	23.3	0.6	61.4	**0.03**	NA	NA
HSC n = 1	88.5	84.7	99.2	10.3	78.5	4.0	**10.7**	89.4	NA
HSC plus $CD8^+TCR^-$ n = 4	82.0 ± 19.4	67.0 ± 23.1	99.0 ± 0.6	6.0 ± 2.8	92.0 ± 10.0	29.0 ± 26.6	**55.0 ± 37.4**	49.5 ± 39.7	$p = 0.1387$
HSC plus $CD8^+TCR^+$ n = 7	98.0 ± 2.4	44.0 ± 9.4	99.0 ± 0.5	28.0 ± 5.8	100.0 ± 0.2	32.0 ± 10.0	**94.0 ± 5.0**	6.8 ± 5.9	$p = <0.0001$
HSC plus $CD8^{tot}$ n = 8	99.0 ± 0.4	56.0 ± 18.0	100.0 ± 0.2	18.0 ± 10.7	93.0 ± 17.9	30.0 ± 12.4	**95.0 ± 10.1**	1.6 ± 1.7	$p = <0.0001$

BALB/c mice are lethally irradiated and allogeneically reconstituted with 250 HSC, 250 HSC + 10,000 $CD8^+$ TCR^- cells, 250 HSC + 15,000 $CD8^+TCR^+$ and 250 HSC + 20,000 $CD8^{tot}$ cells. Figure 3 shows survival data from a series of such experiments. The table shows reconstitution analysis of surviving mice at 8 weeks. The percent of total $CD3^+$ cells and the percent of $CD3^+$ cells that are of HSC origin are markedly reduced in mice reconstituted with HSC alone as compared to groups receiving HSC and $CD8^+$ cells. The last two columns show the statistical analysis of percentage of $CD3^+$ cells that are host derived. Differences are significant between mice reconstituted with HSC alone and mice reconstituted with HSC + $CD8^+TCR^+$ and HSC + $CD8^+TCR^{tot}$ cells.

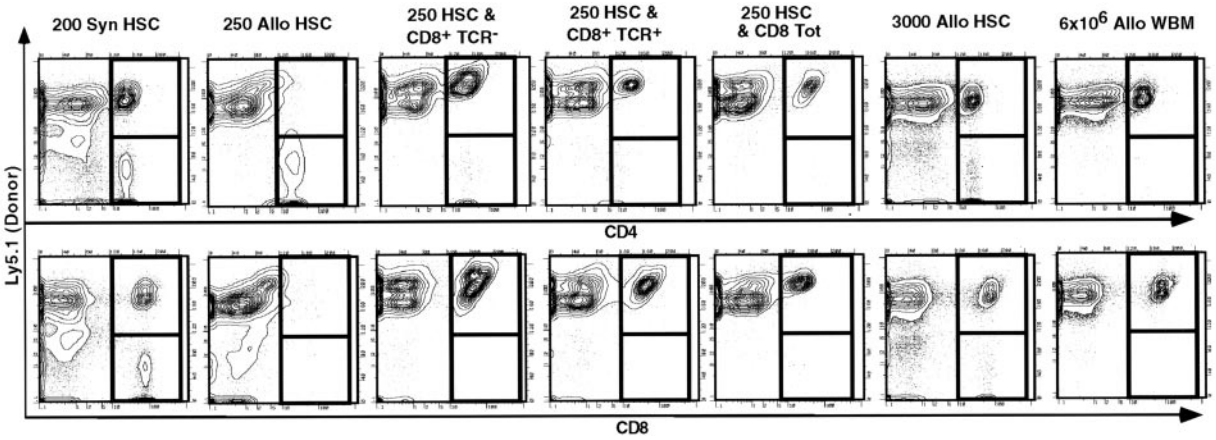

Figure 3. Representative Plots from Lethally Irradiated Mice Reconstituted as Indicated and Analyzed on the FACS >4 Months after Reconstitution

Analysis shows live gated (FSC, SSC, and PI) cells.

origin in the KTLS alone group, >92% of $B220^+$ cells are of donor origin in mice reconstituted with KTLS and any of the CD8 populations. More strikingly, only 10.7% and 55% ± 37.4% of $CD3^+$ cells are of HSC origin in the KTLS alone or KTLS + $CD8^+TCR^-$groups, respectively, whereas nearly all $CD3^+$ cells are donor-derived when KTLS + $CD8^+TCR^+$ (94% ± 5.0%) or KTLS + $CD8^{tot}$ (95% ± 10.1%) cells are used. Related data showing lymphocyte analysis at a later time point are shown in the plots of representative animals in Figure 3. Minimal HSC-derived $CD4^+$ and $CD8^+$ populations are detected in the mice reconstituted with allogeneic KTLS alone, while very distinct populations are detected in mice receiving allogeneic KTLS and the various $CD8^+$ populations (HSC-derived T cells are in the upper right corners of the plots).

Another way of viewing the data is to evaluate the host-derived T cell populations that persist in irradiated hosts (lower right corners of the plots in Figure 3). The host-derived CD3 reconstitution profiles at 8 weeks are analyzed statistically in Table 1; the differences in host-derived $CD3^+$ populations between mice reconstituted with HSC + $CD8^+TCR\beta^+$ and HSC + $CD8^{tot}$ as compared to mice receiving HSC alone is highly significant ($p < 0.0001$). As demonstrated previously, fewer host-derived $CD4^+$ and $CD8^+$ cells persist in lethally irradiated mice after reconstitution with BM (6×10^6) as compared to reconstitution with comparative amounts of purified HSC (3,000) (Gandy and Weissman, 1998), a finding suggested previously by analysis of the total $CD3^+$ population (Shizuru et al., 1996). Comparative analysis of the reconstitution profiles from Figure 3 demonstrates that this loss of host-derived populations is also detected in the mice receiving purifed HSC and $CD8^+$ cells (TCR^-, TCR^+, or both), implying that one effect of facilitator cells is to eliminate or inhibit the persistence of radioresistant host T cells.

Table 2. Flow Cytometric Analysis of White Blood Cell Populations in Control Mice and Lethally Irradiated and Reconstituted Mice

30 Days	Blood Cell Counts $10^3/\mu l$	Percent B220 $Ly5.1^+$	Percent Mac-1 $Ly5.1^+$	CD4 Percent of Total	Percent CD4 $Ly5.1^+$	CD8 Percent of Total	Percent CD8 $Ly5.1^+$
Control	7.0 ± 1.9	NA	NA	23.0 ± 1.3	NA	14.1 ± 0.4	NA
3,000 HSC (Ly5.1) and 30,000 $CD8^+$ (Ly5.2)	6.9 ± 0.7	98.0 ± 2.3	99.7 ± 0.1	25.7 ± 8.1	56.7 ± 35.5	6.5 ± 1.9	66.1 ± 27.3
6×10^6 WBM ($Ly5.1^+$)	5.7 ± 1.1	98.4 ± 1.9	99.6 ± 0.2	19.4 ± 6.6	99.3 ± 0.7	5.5 ± 1.8	99.4 ± 0.5

>4 Months	CD4 Percent of Total	Percent CD4 $Ly5.1^+$	CD8 Percent of Total	Percent CD8 $Ly5.1^+$
C57Bl/Ka Control	32.9 ± 4.0	99.2 ± 1.2	17.5 ± 2.0	98.3 ± 0.9
BALB/c Control	59.7 ± 5.1	0.06 ± 0.04	14.2 ± 1.5	0.1 ± 0.1
3,000 HSC (Ly5.1) and 30,000 $CD8^+$ (Ly5.2)	22.6 ± 6.6	80.5 ± 13.2	16.8 ± 4.5	87.9 ± 8.6
6×10^6 WBM ($Ly5.1^+$)	19.3 ± 4.9	79.6 ± 44.5	12.3 ± 2.0	95.0 ± 7.0
3,000 HSC ($Ly5.1^+$)	18	81.0 ± 4.7	10.0	89.0 ± 2.4

Thirty days or more than 4 months after reconstitution. Host mice are BALB/c. HSC (Ly5.1), WBM (Ly5.1), and $CD8^+$ (Ly5.2) cells are C57BL/Ka derived. Results are given as percentage of total nucleated cells (percent of total) or as percentage of HSC or WBM-derived cells ($Ly5.1^+$).

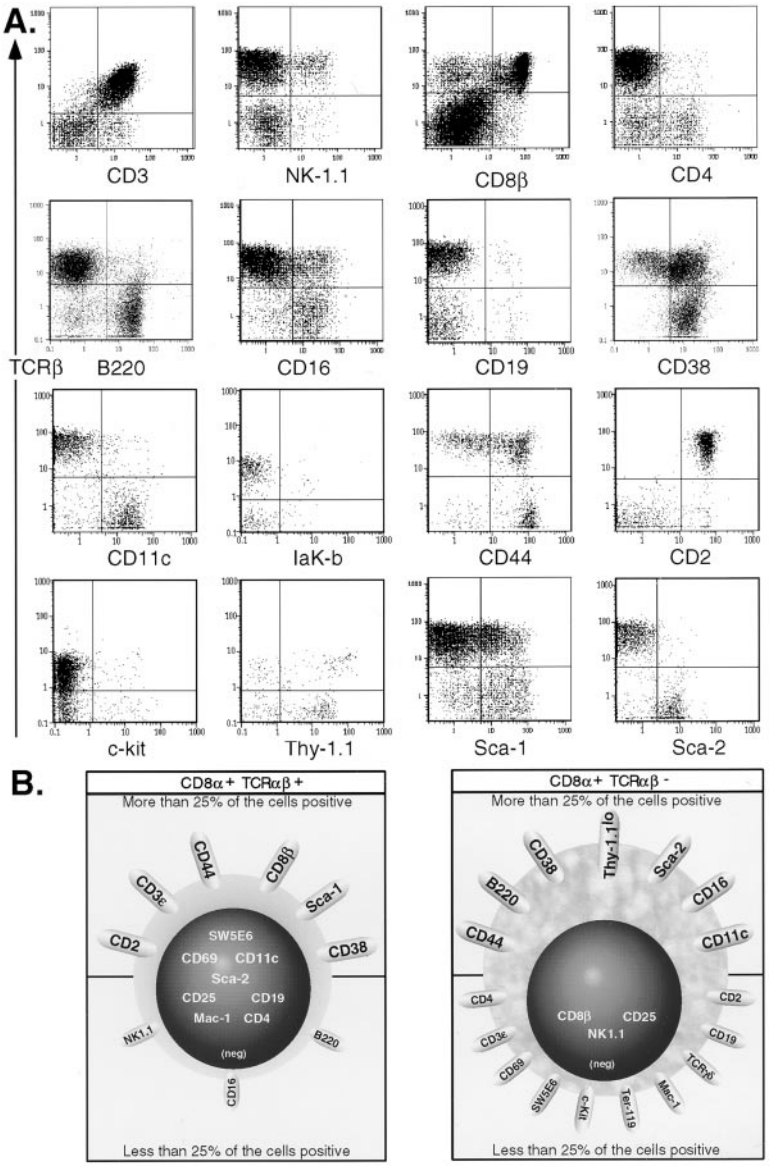

Figure 4. Surface Marker Characteristics of CD8α^+ Cells in BM

(A) C57BL/Ka BM was stained with CD8α, TCRβ, and one other marker. The analyses show staining profiles of approximately 1–5 $\times$ 10^5 CD8$^+$ cells.

(B) Summary of CD8$^+$TCR$^-$ and CD8$^+$TCR$^+$ cell surface phenotypes shown in (A).

The Effect of Facilitator Cells Is Less Pronounced at Higher Dosages of HSC

In a separate experiment, BALB/c mice were reconstituted with 3,000 C57BL/Ka, Thy-1.1 KTLS HSC, and 30,000 CD8$^+$ cells, and reconstitution was compared to that found in mice reconstituted with 6×10^6 BM. Cell counts 35 days after transplantation demonstrated that PBMC had reached normal levels in mice reconstituted with HSC plus facilitators or BM (Table 2). PBMC counts in mice reconstituted with high doses of KTLS alone do not approach normal levels until at least 6 weeks post-transplantation. The percentage of CD4$^+$, CD8$^+$, B220$^+$, or Mac-1$^+$ cells also approached normal levels. Over 98% of these B220$^+$ and Mac-1$^+$ cells were of donor HSC origin in both groups. The percentage of CD4 and CD8 cells that were HSC derived, however, was only 56.8% and 66.1%, respectively, in the group reconstituted with HSC and facilitators and 99.3% and 99.4% in the group reconstituted with BM. By 6 months post-transplantation, donor-derived T cells had increased in the group reconstituted with HSC and facilitators, although the level was little different from mice reconstituted with high levels of HSC alone.

The CD8$^+$TCR$^-$ and CD8$^+$TCR$^+$ Populations Are Characterized by Distinct Cell Surface Markers

Analyses of cell surface markers were performed on unenriched populations to avoid skewing during selection. Data were collected on 1–5 $\times$ 10^5 CD8α^+ events per analysis. Figure 4 shows the cell surface marker patterns of CD8α^+ gated cells. Both the CD8α^+TCRβ^+ and the CD8α^+TCRβ^- subpopulations are still heterogenous for other markers. The CD8α^+TCRβ^+ cells are CD3ϵ^+, CD2$^+$ and most are CD8β^+ and CD44$^+$. They are also CD4$^-$, CD11c$^-$, c-Kit$^-$, and CD19$^-$. The CD8α^+TCRβ^+ population is heterogenous for the markers B220, NK-1.1, CD16, CD38, and Sca-1 and contains

a minor subset of IaK^{b+} cells. The CD8α^+TCRβ^- population is CD8β^-, CD11c$^+$, and CD38$^+$, and predominantly Thy-1.1^{lo}, CD3ϵ^-, and c-Kit$^-$.

The percentage of CD8$^+$TCRβ^- cells that is CD3ϵ^+ varies from 2% to 10% of the total CD8$^+$ cells. The true percentage is probably much less than this, as sorting and resorting of this population (one way to distinguish true events from artifacts caused by doublets, etc.) demonstrated that it was very difficult to isolate CD8α^+TCRβ^-CD3ϵ^+ cells in appreciable numbers, with resorting yields consistently less than 20%. This inconsistent retrieval prevented further subdivision with antibodies to markers such as TCRγ or -δ chains. Although the morphology of CD8α^+TCRβ^- cells included large granular as well as small round cells, the population was NK-1.1$^-$; the majority of these cells had a "lymphoid" dendritic cell phenotype in that they were mainly CD11c$^+$ and FcR (CD16)$^+$ (Wu et al., 1996; Vremec and Shortman, 1997). Because some cells were CD16$^+$, the 24G2 antibody that blocks Fc binding was used in all stains except the 24G2 stain itself. Unfortunately, the rarity of most subsets of the CD8α^+TCRβ^+ and CD8α^+TCRβ^- populations precluded their use in tests of allogeneic HSC facilitation.

Facilitation from CD8 Cells Deficient in Lytic Activity

IL-2-triggered CD8$^+$ T and NK cells proliferate and secrete cytokines such as TNFα and interferon-γ, and a subset goes on to cytolytic function. Cytolytically active NK and CD8$^+$ T cells express the functional proteases granzyme A (GrA) and perforin (Gershenfeld and Weissman, 1986; Hershberger et al., 1992). Transgenic mice expressing the diptheria toxin A chain under the control of the GrA promoter (GrA-DTA) produce CD8 T cells and NK-1.1 cells that are deeply impaired in cytolytic function, and the founderline used here has marked reductions in the number of CD8$^+$ cells in the periphery (Aguila et al., 1995). However, these mice retain full barrier function to transplantated allogeneic HSC (Aguila and Weissman, 1996). CD8$^+$ cells were sorted from the BM of these mice and evaluated for their ability to enhance the engraftment of 250 KTLS across allogeneic barriers. Transgenic CD8$^+$ cells retained this potential (Figure 5A). Survival was not different between mice reconstituted with wild-type CD8^{tot}, GrA-DTA CD8^{tot}, GrA-DTA CD8$^+$TCR$^+$, and GrA-DTA CD8$^+$TCR$^-$, though fewer wild-type CD8^{tot} cells were used. These studies suggest that lytic potential is not necessary for facilitation.

Bone Marrow from CD8$\alpha^{-/-}$ Mice Is Depleted of Facilitative Potential

Because CD8α^+ subsets with facilitative potential are heterogenous with respect to other cell surface markers and morphology, it was reasonable to test the hypothesis that the CD8α (and/or CD8β) molecules themselves played a critical role in facilitation. We tested whether bone marrow from CD8$\alpha^{-/-}$ mice (which also do not express CD8β [Fung-Leung et al., 1991]) could facilitate. As shown in Figure 5B, C57Bl Thy-1.1 CD8$\alpha^{-/-}$ bone marrow is at least 4–5 times less efficient at radioprotecting lethally irradiated BALB/c mice than wild-type marrow. While it is possible and likely that the CD8α molecule is required for the differentiation of bone marrow facilitator cells as opposed to or in addition to being involved directly, the bone marrow from these mice contained both TCRβ^+CD3ϵ^+ (CD4$^-$) cells and TCRβ^-CD3ϵ^- CD11c$^+$IaK^{b+} cells in numbers approaching these subsets in wild-type marrow. The number of stem cells present in CD8$^{-/-}$ BM was analyzed to exclude the possibility that the decreased survival was due to fewer HSC in a given amount of BM. As can be seen in Figures 5C and 5D, there are no fewer HSC in CD8$^{-/-}$ BM as determined by by day 12 CFU-S assay and cell surface phenotype.

Discussion

Description of the Model

Detection of facilitation is dependent upon the model. These studies evaluated engraftment of hematopoietic cells across both major and minor histocompatibility barriers (C57BL/Ka-Thy1.1→BALB/c) in recipients that received a lethal dose of irradiation. It has been previously determined that some T cells and NK cells persist in hosts after this conditioning regimen, although the number of T cells is reduced 100- to 1,000-fold (Domen et al., 1998). Therefore, facilitation is being evaluated in a model with persistent T and NK cells, both of which have been shown to mount a barrier to engraftment (Dennert et al., 1985; Murphy et al., 1987; Schwartz et al., 1987; Tiberghien et al., 1990; Blazar et al., 1991; Hiruma et al., 1992; Shizuru et al., 1996). The model used here was designed such that progeny derived from facilitator, HSC, and host could be distinguished. Most reported models do not distinguish between cells of donor-HSC and facilitator origin. This distinction is important in the evaluation of T cell recovery. This is also the first reported analysis of allogeneic engraftment of rigorously purified HSC (KTLS) in the context of facilitation. In this model, facilitation was evaluated by three different parameters: survival, enhancement of recovery of total donor-derived populations, and facilitated removal (or loss) of residual host lymphoid cells.

The quantitative analysis of the minimal number of CD8^{tot} cells (and the TCRβ^- and TCRβ^+ subsets) required for facilitation of 250–500 cotransplanted HSC underscores a point made in three previous publications (Shizuru et al., 1996; Gandy and Weissman, 1998; Uchida et al., 1998): the dose of purified HSC that is both fully radioprotective and fully engrafting in the C57BL/Ka→BALB/c transplant, i.e., 1,000–6,000 cells, does not engraft due to contaminating facilitator cells, since we show here that the minimal dose of any CD8$^+$ (or CD8$^+$ + Mac-1$^+$, for that matter) population capable of facilitation of 250–500 HSC is 15–30 $\times$ 10^3 cells.

Most Facilitator Potential Is Contained in the CD8$^+$ Population

This study was designed to detect the most efficient facilitator populations in BM at physiological numbers. Initially, cells of various subpopulations were added in the numbers present in 1 $\times$ 10^6 BM, which are different for each population. The CD8$^+$ population, unfractionated for TCRβ expression, was the most efficient in facilitating both radioprotection and HSC engraftment

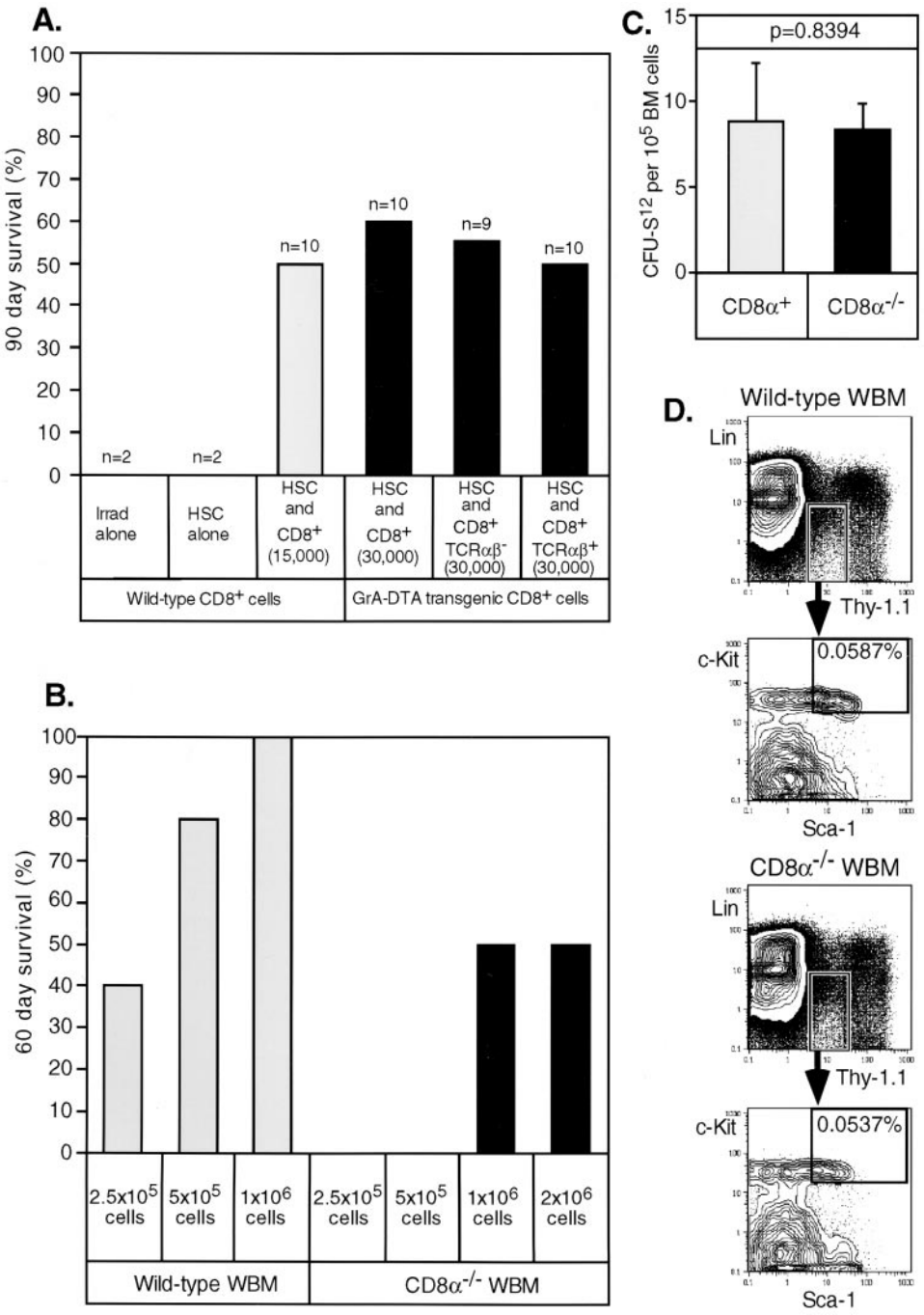

Figure 5. Facilitator Cells from Mutant Mice

(A) GrA-DTA mice. Survival at 90 days in lethally irradiated BALB/c mice reconstituted with 250 HSC and $CD8^+$ populations as indicated. (B–D) $CD8^{-/-}$ mice (B). Survival at 60 days in lethally irradiated BALB/c mice reconstituted with wild-type or $CD8^{-/-}$ BM. (C) Analysis of CFU-S day 12 activity of 10^5 wild-type BM or 10^5 $CD8^{-/-}$ BM. (D) Analysis of frequency of KTLS HSC by staining for surface markers.

of those analyzed. Analysis of these data suggests, however, that some facilitative potential was present outside of the $CD8^+$ population. Although analysis of Mac-1^+ populations by Fisher's exact test were not statistically significant, analysis of survival by Kaplan-Meier statistics did show significant results. It is of note, however, that the facilitation found with transfer of the Mac-1^{tot} populations was lost when the Mac-1^{hi} subpopulation was used as opposed to the Mac-1^{hi} and Mac-1^{lo} combined. Because short-term HSC are Mac-1^{lo} (Morrison and Weissman, 1994), it is conceivable that contaminating HSC account for this increased radioprotection. This finding is reminiscent of several previous studies in which some degree of facilitation was reported in non-$CD8^+$ populations (Murphy et al., 1992; Martin, 1993; Drobyski and Majewski, 1997). It suggests that at limiting

dosages of HSC, multiple populations may facilitate. One might question whether an increase in the number of CD4 cells may improve facilitation. This, however, is not the case, as survival was not improved in mice given up to 80,000 $CD4^+$ cells (data not shown).

The $CD8^+TCR^+$ and $CD8^+TCR^-$ Subpopulations in Whole Bone Marrow Significantly but Incompletely Facilitate HSC Engraftment

Both the TCR^+ and TCR^- subpopulations of $CD8^+$ BM can facilitate HSC engraftment. This contrasts with the studies presented in Kaufman et al. (1994) in which the $CD8^+TCR^+$ population of BM was not found to enhance engraftment, but is in accordance with those of Deeg, Martin, and Lapidot (Deeg et al., 1979; Lapidot et al., 1992; Martin, 1993), in which predominantly TCR^+ populations enhance HSC engraftment. The facilitative potential found in the TCR^+ cells in this study cannot be attributed to a contaminating TCR^- population: the purity of the TCR^+ populations used in these experiments was >95%. Even if every contaminating cell in the TCR^+ populations were a $CD8^+TCR^-$ cell (which it was not), the numbers would not approach the threshold number necessary to detect facilitation. It is of note that the number of $CD8^+TCR^+$ cells used in this study was lower than that used by Kaufman et al. (1994) and that our facilitator populations were cotransplanted with rigorously purified KTLS HSC. Finally, our studies utilized different mouse strains than the ones used in Kaufman et al. (1994), and these strain variations might contribute to the differences.

The Surface Phenotype of the $CD8^+TCR^-$ Cells in This Study Differs from Other Studies

The majority of the $CD8^+TCR^-$ cells in this study were $CD3^-$. There was a small population of $CD3^+$ cells in the $CD8^+TCR^-$ cells, but these could not be obtained in significant numbers, especially since multiple sorts were necessary for purification. This result has been confirmed by staining with CD3ϵ antibodies originating from different hybridomas and by staining with CD3ϵ antibodies conjugated to a variety of different fluorochromes. The type of CD8 molecules on the surface of these cells is also noteworthy. No CD8β is detected by flow cytometry. These cells are probably CD8αα, a surface phenotype reminiscent of a population of non-T cells, including lymphoid dendritic cells as characterized in the Shortman lab (Wu et al., 1996; Vremec and Shortman, 1997). Because these cells are also $Fc\gamma R^+$, false-positive staining by adhesion of Ig molecules via their Fc rather than F(ab) regions is a real possibility. This was prevented by blocking with the anti-FcR mAb 24G2.

CD8α Is Important for Facilitation

The studies that demonstrate preservation of facilitative function in CD8 cells from the GrA-DTA mice suggest that lytic function is not critical. The fact that the CD8α molecule was present on heterogeneous populations of BM that had facilitative potential prompted the analysis of CD8α in this system. CD8 coreceptors have been shown to play a role in the interaction of the TCRαβ complex with its MHC class I targets, perhaps by increasing the avidity of cell–cell interactions (Norment et al., 1988; Rosenstein et al., 1989; Ashton-Rickardt et al., 1994; Luescher et al., 1995), as well as via signal transduction mechanisms (Rudd, 1990; Rudd et al., 1991; Cai and Sprent, 1994). Mice lacking the CD8α molecule have been described (Fung-Leung et al., 1991). Here we demonstrated that facilitation was markedly compromised when BM from these mice was used as a source of facilitators. Recent data has provided evidence that the CD8 complex is important in vitro in the veto effect and that cross-linking of CD8 in vitro induces secretion of TGFβ1, which may lead to apoptosis of activated alloreactive cells (Asiedu et al., 1999). More studies are needed to further assess the role of CD8α and its MHC class I target in facilitation.

A Major Function of $CD8^+$ Facilitator Cells Is to Eliminate Residual Host T Cells

In earlier engraftment comparisons between a given amount of BM and the number of HSC contained therein in the C57BL→BALB/c model of allotransplantation, we noted that BM but not HSC eliminated residual host T cells (Shizuru et al., 1996). Here we demonstrate that the elimination of residual host T cells is a property of $CD8^+$ facilitator cells and that $CD8^+TCR^+$ cells are more effective in this respect than $CD8^+TCR^-$ cells. In several fully allogeneic transplantation models, T cells as well as NK cells can participate in barrier functions (Bennett et al., 1987; Murphy et al., 1987; Aguila and Weissman, 1996; Lee et al., 1996; Shizuru et al., 1996). In most studies of parent (e.g., C57BL) to F1 (e.g., [BALB/c × C57BL]F1) engraftment, NK cells appear to be the predominant cellular mediator of barrier function (Aguila and Weissman, 1996; Shizuru et al., 1996; Kumar et al., 1997), although the effect of T cells has been clearly noted (Davenport et al., 1995). In both parent-to-F1 and P1-to-P2 allogeneic transplants, the barrier cells do not require granule-mediated (Baker et al., 1995; Aguila and Weissman, 1996; Graubert et al., 1996) or FasL-mediated (Baker et al., 1995) lytic capacity. Martin et al. have previously demonstrated that the absence of perforin but not the absence of granzyme B significantly altered function of $CD8^+$ facilitator cells in a model of BM transplantation (Martin et al., 1998). They similarly demonstrated that the combination of deficiencies in FasL and granzyme B significantly reduced facilitative function. In our experiments, some facilitation is preserved in the absence of cells that operate via both granzyme and perforin function. Thus facilitation can occur without these cells. Due to the close temporal regulation of granzyme A, granzyme B, and perforin, activated T cells in the GrA-DTA transgenic mice express diphtheria toxin A and undergo apoptosis at the time of expression of these molecules. There is an apparent discrepancy between lost perforin function leading to lost facilitator function (Martin et al., 1998), and elimination of activated $CD8^+$ GrA^+ cells that express perforin affecting facilitation far less (this study). Perhaps the use of limiting numbers of HSC in allogeneic transplants allows facilitation to be detected at more subtle levels. Alternatively, perforin deletion and GrA-DTA transgenes could result in differences in T cell development that prevent the

development of facilitators in the former but not in the latter. Operationally, facilitation resembles the veto phenomenon, wherein MHC-bearing $CD8^+$ cells "veto" the alloreactive populations of effector cells and in so doing enable the development of MHC-allele-specific tolerance (Heeg and Wagner, 1990; Thomas et al., 1994). Candidate effector mechanisms for the functions of barrier cells, facilitator cells, and veto cells should include alternative cell death pathways such as those mediated by LT or TNF (Ware et al., 1996), TGFβ1 (Asiedu et al., 1999), and maybe even those found in early (proto)chordates (Rinkevich and Weissman, 1992). The experiments reported here now demand a careful comparison of at least four classes of cells—barrier cells, veto cells, facilitator cells, and lymphoid dendritic cells—all of which share at least some markers. Such a comparison should include analyses of the lineage relationships, their effector mechanisms, and possibly their overlapping identities.

Clinical Implications

The importance of facilitation is likely dependent on the context in which it is evaluated. Facilitation in the context of reconstitution with limiting numbers of HSC could be clinically relevant and is the subject of this study. The importance of facilitation in the context of reconstitution with large numbers of hematopoietic cells is more controversial. It has been determined that HSC alone as purified by several methods are sufficient for engraftment across allogeneic barriers. Shizuru et al. first demonstrated that TLS HSC could fully radioprotect and engraft lethally irradiated hosts disparate at either or both major and minor histocompatibility loci and that the number of HSC required increased with the degree of histocompatibility disparity (Shizuru et al., 1996). Other studies utilizing the additional c-Kit marker (KTLS) in C57Bl→BALB/c mice have determined that as few as 1,000 HSC can save 95%–100% of lethally irradiated hosts and 10^4 KTLS HSC lead to rapid (day 11) engraftment of WBC and platelets, even if the more primitive $Rh123^{lo}$ subset of KTLS HSC is used (Uchida et al., 1998). The results of these experiments can be affected by the condition of the mouse colony. Within the course of the experiments presented here, the condition of the mice improved such that 500 HSC, a dose which would originally only save 20% of mice, radioprotected 40%–80% (Gandy and Weissman, 1998). Engraftment efficiency of BM improved similarly. The three studies just described evaluated allogeneic engraftment of HSC that contained both long- and short-term progenitors. Wang et al. recently reported engraftment of a population characterized in a different manner that also lacks facilitators ($Lineage^-$, $CD71^-$, MHC class I highly positive cells from 5-FU-treated mice) and which is purported to contain only long-term progenitors (Wang et al., 1997). Although these studies all confirm that purified HSC are sufficient to overcome the allogeneic barrier when used in large numbers, the extrapolation of allogeneic HSC ± facilitator transplants in humans of differing histocompatability disparities is, at this point, hypothetical. If the HSC dose that will be available clinically is at or near the limiting dilution for survival, then putative human facilitator cells may be useful if not vital.

Experimental Procedures

Mice

BALB/c ($H\text{-}2^d$); C57BL/Ka, Thy-1.1, Ly-5.2 ($H\text{-}2^b$); and C57BL/Ka, Thy-1.1, Ly-5.1 ($H\text{-}2^b$) mice were maintained in the animal care facilities at Stanford. Mice were given acidified water for at least 1 week prior to irradiation and antibiotic water (1.1 g/L neomycin sulfate and 10^6 U/L polymixin B sulfate) for at least 8 weeks after BM transplant to reduce the chance of infection. GrA-DTA transgenic mice were created in our lab as previously described (Aguila et al., 1995). Hematopoietic donors were 4–6 weeks of age; recipients were 7–12 weeks of age. For reconstitution, cells to be injected were mixed and resuspended in 100–200 μl PBS prior to retroorbital i.v. injection.

Antibodies for Cell Sorting and Analysis

The rat antibodies 53-7.3 (anti-CD5), 53-6.7 (anti-CD8), Ter-119 (anti-erythrocyte specific antigen), GK 1.5 (anti-CD4), KT 31.1 (anti-CD3), 6B2 (anti-B220), M1/70 (anti-Mac-1), 8C5 (anti-GR-1), 19XE5 (anti-Thy-1.1), E13–161 (anti-Sca-1), A20.1.7 (mouse anti-Ly5.1), and 2B8 (anti-c-Kit) were prepared from the respective clones. Antibodies were conjugated with FITC, PE, or APC. The following conjugates were used: KT 31.1-PE, 6B2-FITC, M1/70-FITC, 8C5-PE, 19XE5-FITC, A20.1.7-APC, and 2B8-APC. Goat anti-rat IgG-PE was obtained from Caltag (catalog number R40004–3). Avidin Texas red (avidin-TR, catalog number 55894) was obtained from Cappel.

Stem Cell Sorting

HSCs were prepared as previously described. Briefly, BM was obtained from the tibias and femurs of adult mice. Cells were incubated with antibodies against lineage markers (53-7.3, 53-6.7, Ter-119, GK 1.5, KT31.1, 6B2, M1/70, and 8C5), washed with staining media (1× HBSS with 3% FCS and 10 mM HEPES [pH 7.0]), and incubated with GAR-IgG-PE (30 min), followed by incubations with rat IgG (to occupy any nonspecific binding sites) (10 min), anti-Sca-1 (20 min), avidin-MiniMacs beads (20 min), and anti-Thy-1.1-FITC and avidin-TR with or without anti-c-Kit-APC (20 min). Cells were passed twice through Minimacs columns (Miltenyi Biotec). Retained cells (5- to 10-fold enriched for HSC activity) were eluted and resuspended in propidium iodide (PI)-containing media.

$CD8^+$ Cell Sorting

BM was incubated with 53-6.7-biotin (20 min). Cells were then washed and incubated with avidin-MiniMacs beads (20 min). Cells were washed and passed through a MiniMacs column as described. Cells retained in the column were eluted and incubated with AV-PE (Caltag) or AV-TR (Pharmingen) (20 min). Cells were washed, resuspended in PI, and sorted. For sorting of the $TCR\beta^+$ and $TCR\beta^-$ subsets of the $CD8\alpha^+$ cells, cells were additionally stained with TCRβ-APC (gift of the lab of Sam Strober) or TCRβ-FITC (Pharmingen) after elution from the collumn. Cells were then sorted with the gates shown in Figure 2, 10 chanels in the TCRβ axis between the two populations.

Strains of Mice Used for Reconstitution

The experimental design allows for evaluation of the origin of each of the lineages. HSC and CD8 donor mice were C57Bl/Ka Ly5 congenic sublines, and recipients were BALB/c. C57Bl/Ka mice are $H\text{-}2^b$ haplotype, and BALB/c recipients are $H\text{-}2^d$. Cells of donor facilitator origin could be differentiated from donor HSC origin by the congenic Ly5 marker. Figure 1 shows how this method can be used to differentiate cells. Using this system, cellular progeny can be evaluated for an additional two markers on a two-laser, five-color modified FACS Vantage, made available through the Stanford Shared FACS facility. $CD8\alpha^{-/-}$ mice were generated in the lab of Tak Mak and were obtained through Jane Parnes, Stanford. These mice were backcrossed 3–8 times to C57BL/Ka, Thy-1.1 mice and screened for the appropriate Ly5 marker for use in these experiments.

Evaluation of TCRβ Gene Rearrangement

Analysis of TCRβ rearrangement (Db1.1 to Jb1.7 and Db2.1 to Jb2.7) by PCR was done as described (Mebius et al., 1997) on 10,000 cells sorted directly into 0.5% Tween D in PCR buffer. The PCR was done as follows: 9 cycles with the external primers (30 s, 94°C; 2 min,

63°C; 3 min, 72°C) and 25 cycles with the internal primers (30 s, 94°C; 1 min, 63°C; 3 min, 72°C).

Gross Analysis

All mice were weighed every week or every other week. Upon weight loss of greater than 30% of original weight, animals were sacrificed and tissue taken for immunohistochemistry.

Analysis of Reconstitution

Reconstituted animals were bled 2–12 weeks after reconstitution for analysis. PBMC were collected in 5 mM EDTA in PBS. In some procedures, cells were then separated in a dextran gradient and residual red cells were lysed with 0.15 M ammonium chloride/0.01 M potassium bicarbonate. In other procedures, no dextran gradient was used and red cells were lysed in the ammonium chloride solution without the intervening step. Cells were divided into two or three aliquots and stained with the following antibody panels: A20.1.7-APC, 6B2-FITC, KT-31.1-PE; A20.1.7-APC, M-170-FITC, 8C5-PE; A20.1.7-APC, GK-1.5-PE, 53.67-FITC; A20.1.7-APC, NK-1.1-PE, 6B2-FITC; A20.1.7-APC, $H2K^b$-Bio, GK-1.5-PE, 53-6.7-FITC. Cells were resuspended in PI-containing staining media for FACS analysis. Antibodies were titered such that haplotype controls had less than 1% nonspecific staining. Donor chimerism is not corrected for such nonspecific staining. Consecutive bleedings were at least 4 weeks apart.

Statistics

Statistical analysis was performed using Fischer's exact test. Values are reported as p values from this analysis, in which the experimental value was compared with that of the control group (i.e., HSC alone). In one experiment, further analysis of survival was performed using the Kaplan-Meier method, which analyzes the data over a cross-section in time and is more sensitive. The authors used the former test to determine the course of future studies.

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